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Peters, K., Marie, J., Wilson, E., Ives, H., Escobedo, J., Del Rosario, M., Mirda, D. and Williams, L. (1992). Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca.sup.2+ flux but not mitogenesis. Nature 358, 678-681.

Thanks a lot...

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## Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and $\text{Ca}^{2+}$ flux but not mitogenesis

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STIMULATION of certain receptor tyrosine kinases results in the tyrosine phosphorylation and activation of phospholipase  $\text{C}_\gamma$  (PLC $\gamma$ ), an enzyme that catalyses the hydrolysis of phosphatidylinositol (PtdIns)<sup>1-8</sup>. This hydrolysis generates diacylglycerol and free inositol phosphate, which in turn activate protein kinase C and increase intracellular  $\text{Ca}^{2+}$ , respectively. PLC $\gamma$  physically associates with activated receptor tyrosine kinases, suggesting that it is a substrate for direct phosphorylation by these kinases<sup>7-10</sup>. Here we report that a fibroblast growth factor (FGF) receptor with a single point mutation at residue 766 replacing tyrosine with phenylalanine fails to associate with PLC $\gamma$  in response to FGF. This mutant receptor also failed to mediate PtdIns hydrolysis and  $\text{Ca}^{2+}$  mobilization after FGF stimulation. However, the mutant receptor phosphorylated itself and several other cellular proteins, and it mediated mitogenesis in response to FGF. These findings show that a point mutation in the FGF receptor selectively eliminates activation of PLC $\gamma$  and that neither  $\text{Ca}^{2+}$  mobilization nor PtdIns hydrolysis are required for FGF-induced mitogenesis.

It has been shown that the FGF receptor can phosphorylate itself on Tyr 766 and that a recombinant SH2 domain (src-homologous domain 2) of PLC $\gamma$  can bind to a tryptic fragment of the receptor containing this tyrosine<sup>11</sup>. We reasoned that if Tyr 766 were the main site of interaction between the FGF receptor and PLC $\gamma$ , a point mutation at this residue might produce a receptor with a selective impairment in the PtdIns turnover pathway. To determine the specificity of the interaction between the FGF receptor and PLC $\gamma$  we synthesized peptides encompassing the two conserved tyrosines in the C-terminal tail of the receptor, Tyr 766 and Tyr 776, and asked whether they could block the association of PLC $\gamma$  with recombinant FGF receptors *in vitro*<sup>12-14</sup>. A peptide of nine amino acids that was phosphorylated on Tyr 766 (Y766P) blocked the association of

PLC $\gamma$  with the FGF receptor at 10  $\mu\text{M}$ , the lowest concentration tested (Fig. 1). The unphosphorylated version of this peptide (Y766U) partially blocked PLC $\gamma$  association but only at much higher concentrations (200  $\mu\text{M}$ ). A peptide phosphorylated at Tyr 776 failed to block PLC $\gamma$  association (data not shown). A scrambled phosphopeptide (Y766PS) also failed to block PLC $\gamma$  association (Fig. 1). These results suggested that Tyr 766, when phosphorylated, was directly involved in the binding of PLC $\gamma$  to the FGF receptor and that Tyr 766 and its flanking sequences constituted the major site of PLC $\gamma$  association with the FGF receptor.

To test the functional significance of PLC $\gamma$  binding to Tyr 766, we made a mutant FGF receptor in which Tyr 766 was replaced by phenylalanine (Y/F766). The Y/F766 mutant receptor and the wild-type receptor were then transfected into rat L6 myoblasts that do not express endogenous FGF receptors<sup>15</sup>. FGF treatment of cells expressing the wild-type FGF receptor enhanced receptor association of PLC $\gamma$  (Fig. 2a). But in two independent cell lines expressing the Y/F766 FGF receptor (Y/F766-1 and Y/F766-2), there was no detectable ligand-induced receptor association of PLC $\gamma$ , despite the fact that more receptors were immunoprecipitated from these cells (Fig. 2a). More receptor is immunoprecipitated from the Y/F766 cell lines because these cells express around three times more receptor protein than the wild-type cell line (data not shown). We have recently isolated and evaluated an additional Y/F766 mutant cell line that expresses a similar amount of receptor protein as the wild-type clone. In this cell line the mutant FGF receptor behaved the same as in the clones that had more receptors.

Paralleling receptor association, tyrosine phosphorylation of PLC $\gamma$  by the Y/F766 mutant receptor was dramatically decreased or absent compared with the wild-type receptor and there was no increase in PtdIns turnover in response to FGF stimulation (Fig. 2b and c). (A small amount of baseline tyrosine phosphorylation of PLC $\gamma$  was seen in multiple experiments using several cell lines, including another vector-transfected cell line, and probably represents phosphorylation of PLC $\gamma$  by kinases other than the FGF receptor.) These results further establish that Tyr 766 is necessary for ligand-induced association

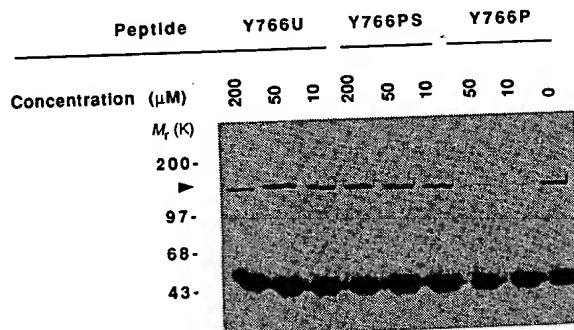


FIG. 1 A synthetic FGF receptor phosphopeptide encompassing Y766 blocks association of PLC $\gamma$  with the FGF receptor tyrosine kinase *in vitro*. Peptides were synthesized based on the sequence of nine amino acids surrounding Y766 of the human FGF receptor<sup>14</sup>. Peptide Y766P (SNQEY<sub>766</sub>LDLS) and a scrambled peptide, peptide Y766PS (Y<sub>766</sub>DSLQSELN), were synthesized using phosphotyrosine; peptide Y766U (SNQEYLDLS) was synthesized using unphosphorylated tyrosine. These peptides were then tested for their ability to block the association of PLC $\gamma$  to recombinant FGF receptors *in vitro*. Arrowhead indicates receptor-associated PLC $\gamma$ .

METHODS. Peptides were synthesized and receptor association was assayed as described<sup>13</sup>. Recombinant chicken FGF receptor produced in a baculovirus expression system was immunoprecipitated from insect cell lysates using a polyclonal FGF receptor antibody (unpublished). The immunoprecipitated receptor was autophosphorylated *in vitro* and incubated with BALB/c 3T3 cell lysates that were preincubated with or without one of the synthetic peptides. The resultant receptor immunoprecipitates were analysed for the presence of co-immunoprecipitated PLC $\gamma$  by immunoblot with anti-PLC $\gamma$  antibodies (UBI).

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of PLC $\gamma$  with the FGF receptor *in vivo* and suggest that receptor association is required for the phosphorylation and activation of PLC $\gamma$ .

Many peptide growth factors, including FGF, stimulate a rapid increase in cytosolic Ca<sup>2+</sup> concentration<sup>16</sup>. This process occurs in two phases, the first by transient release of Ca<sup>2+</sup> from intracellular stores and the second by sustained entry of Ca<sup>2+</sup> from the extracellular space. The release of Ca<sup>2+</sup> from intracellular stores is probably mediated by inositol trisphosphate, and we have recently shown that Ca<sup>2+</sup> entry from the extracellular space might also be mediated by a PtdIns metabolite<sup>17</sup>. In cells expressing the wild-type FGF receptor, FGF stimulated both phases of Ca<sup>2+</sup> mobilization (Fig. 3a). But in cells expressing the Y/F766 mutant receptor, FGF failed to stimulate increased intracellular Ca<sup>2+</sup> from either intracellular stores or the extracel-

lular space (Fig. 3b). Cells expressing either the wild-type or the mutant receptor had a normal Ca<sup>2+</sup> response to platelet-derived growth factor (PDGF) (data not shown). These data strongly suggest that PtdIns turnover is required for FGF-induced Ca<sup>2+</sup> mobilization from either intracellular stores or from the extracellular space.

Despite deficient interactions with PLC $\gamma$ , the Y/F766 FGF receptor mutant was a functional receptor tyrosine kinase capable of responding to ligand stimulation. Both the wild-type and mutant FGF receptors were autophosphorylated in response to FGF (Fig. 4a) and both receptors mediated the phosphorylation of other cellular proteins after FGF stimulation (Fig. 4b, closed arrows). Two proteins (*M*<sub>r</sub> 145,000 and 90,000) even seem

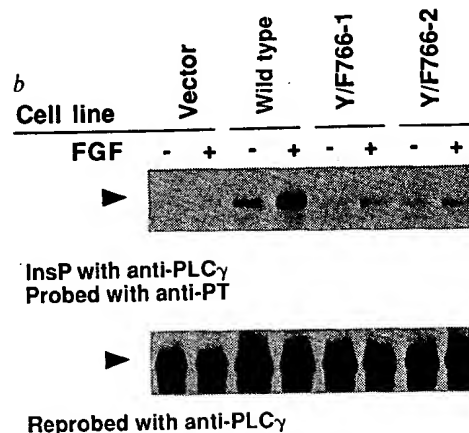
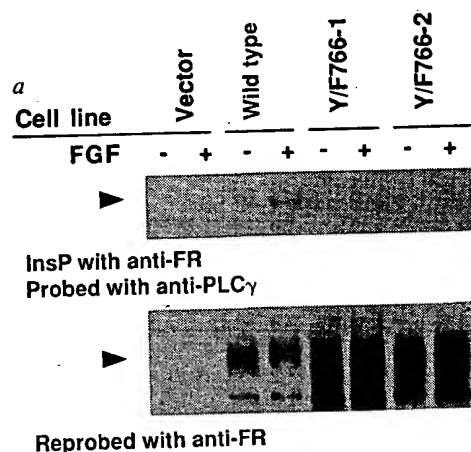
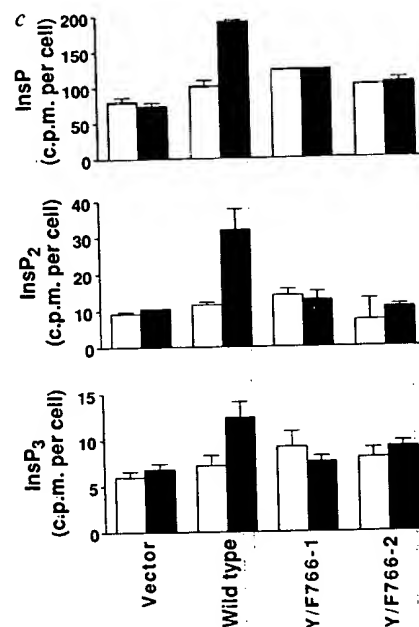


FIG. 2 A mutant FGF receptor with a single point mutation replacing Tyr 766 with phenylalanine (Y/F766) fails to undergo ligand-stimulated association with PLC $\gamma$  and is defective in mediating the tyrosine phosphorylation and activation of PLC $\gamma$  *in vivo*. **a**, Failure of the Y/F766 FGF receptor to undergo FGF-stimulated association with PLC $\gamma$ . Rat L6 myoblasts were transfected with cDNA encoding the wild-type FGF receptor or the Y/F766 mutant FGF receptor (two independent clones, Y/F766-1 and Y/F766-2). To test for the presence of ligand-stimulated association of PLC $\gamma$  with these receptors, receptor immunoprecipitates from both untreated (–) and FGF-treated (+) cells were probed by immunoblot using an antibody against PLC $\gamma$ . Arrowhead in the upper panel indicates PLC $\gamma$  co-immunoprecipitated with the FGF receptor. The blot was reprobed with antibody against the FGF receptor (anti-FR); arrowhead in the lower panel indicates immunoprecipitated FGF receptor. **b**, Mutant FGF receptors defective in PLC $\gamma$  association are also defective in mediating tyrosine phosphorylation of PLC $\gamma$ . Anti-PLC $\gamma$  immunoprecipitates made from unstimulated (–) and FGF-stimulated (+) cells were probed with an antiphosphotyrosine antibody (anti-PT). Arrowhead in the upper panel indicates tyrosine-phosphorylated PLC $\gamma$ . The blot was reprobed with anti-PLC $\gamma$ ; the arrowhead in the lower panel indicates immunoprecipitated PLC $\gamma$ . **c**, Ligand-activated mutant FGF receptors do not mediate increased PtdIns turnover. Open columns, inositol phosphate (InsP) accumulation in unstimulated cells; solid columns, inositol phosphate accumulation in FGF-stimulated cells. Error bars indicate s.e.m. of triplicate samples.

**METHODS.** The FGF receptor mutant (Y/F766) was constructed using the cDNA for the two-immunoglobulin-domain form of the human FGF receptor 1 as the template for site-directed mutagenesis with an oligonucleotide containing a single T/A change that encoded the Y/F766 mutation; ACAGGTCAGGAACCTCTGGTTG (ref. 14). Both the wild-type and the mutant cDNA were then subcloned into a pSV7d expression vector and cotransfected with pSV7d-NEO into rat L6 myoblasts. After G-418 selection, clones expressing mutant or wild-type FGF receptors were identified by antiphosphotyrosine immunoblot or receptor immunoblot. For receptor association experiments, subconfluent cultures of L6 cell transfectants (2–4 15-cm plates) were either left unstimulated or were stimulated with basic FGF (50 ng ml<sup>–1</sup>) for 10 min. After stimulation, cells were lysed in 1% Triton X-100 lysis buffer (1 ml per plate) containing 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, aprotinin (0.15 U ml<sup>–1</sup>), leupeptin (1  $\mu$ g ml<sup>–1</sup>) and 1 mM vanadate for 10 min at 4 °C. Lysates were then incubated overnight with anti-FR. Immune complexes were collected on



protein A-Sepharose, washed three times with 1% Triton lysis buffer and once with 10 mM Tris, pH 7.4, and analysed by 7% PAGE. Immunoprecipitated protein was transferred to nitrocellulose and subsequently probed with anti-PLC antibody. Experiments to detect tyrosine phosphorylation of PLC $\gamma$  were done similarly, except that anti-PLC immunoprecipitates were probed with anti-PT. For PtdIns turnover assays, cells in 6-well plates were incubated in Q-medium (DMEM, 20 mM HEPES, pH 7.4, 1  $\mu$ g ml<sup>–1</sup> insulin, 5  $\mu$ g ml<sup>–1</sup> transferrin and 0.5 mg ml<sup>–1</sup> BSA) with 10  $\mu$ Ci ml<sup>–1</sup> [2-<sup>3</sup>H(N)]myo-inositol for 24 h. After labelling, cells were incubated for an additional 15 min in Q-medium with 20 mM LiCl and then treated with basic FGF (50 ng ml<sup>–1</sup>) or vehicle for 30 min. At the end of 30 min, cells were extracted with acid (10% HClO<sub>4</sub>, 0.5 ml per well). Neutralized extracts were applied to BioRad 1-X8 AG columns (formate form) and free inositol phosphates were eluted in a formate step gradient.

to be phosphorylated more efficiently by the mutant receptor, and at least two additional phosphoproteins were detected after stimulation of the mutant receptor that were not detected after stimulation of the wild-type receptor (Fig. 4b, open arrows). The increased baseline autophosphorylation and the increased phosphorylation of substrates after stimulation of the mutant

receptors may be the result of higher receptor expression, but small differences in the kinase activity between the wild-type and mutant receptors cannot be ruled out. The increased mobility of the ligand-stimulated mutant receptor compared with the wild-type receptor might be secondary to the loss of the Tyr 766 autophosphorylation site or, alternatively, to the loss of phos-

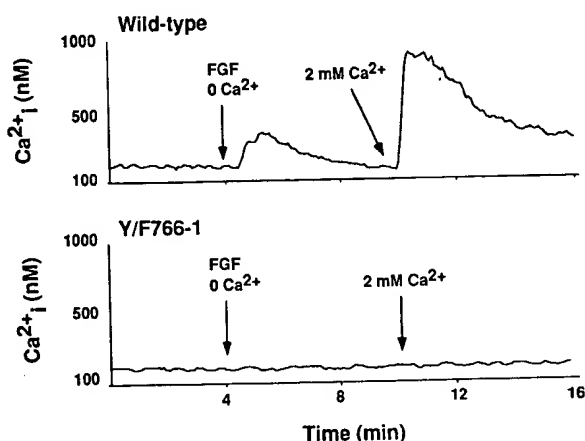


FIG. 3 The Y/F766 mutant FGF receptor does not mobilize  $\text{Ca}^{2+}$  either from intracellular stores or from the extracellular space in response to FGF. To assess  $\text{Ca}^{2+}$  mobilization from intracellular stores, basic FGF ( $5 \text{ ng ml}^{-1}$ ) was added to L6 myoblasts expressing the wild-type FGF receptor (upper panel) or the Y/F766 FGF receptor (lower panel) in  $\text{Ca}^{2+}$ -free buffer containing 10 mM EGTA (left arrow). After 10 min the buffer was changed to  $\text{Ca}^{2+}$ -containing buffer without EGTA (right arrow) to assess the contribution of  $\text{Ca}^{2+}$  entry to calcium mobilization by the wild-type and Y/F766 mutant FGF receptors.

METHODS. Measurement of intracellular  $\text{Ca}^{2+}$  was performed with the fluorescent probe Fura-2 as described<sup>17</sup>. Intracellular  $\text{Ca}^{2+}$  concentrations were calculated by comparing the ratios of fluorescence at 340/380 nm obtained at maximal intracellular  $\text{Ca}^{2+}$  (achieved by addition of  $10 \mu\text{M}$  4Br-A23187) and minimal intracellular  $\text{Ca}^{2+}$  (achieved by addition of 20 mM EGTA).

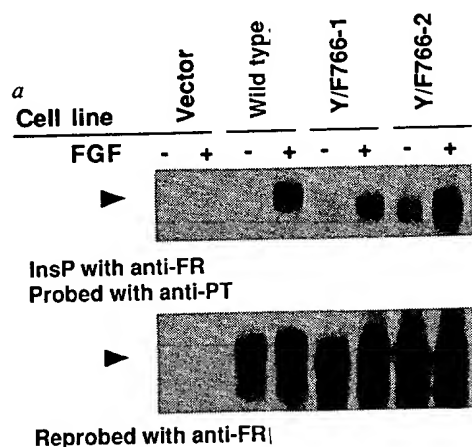
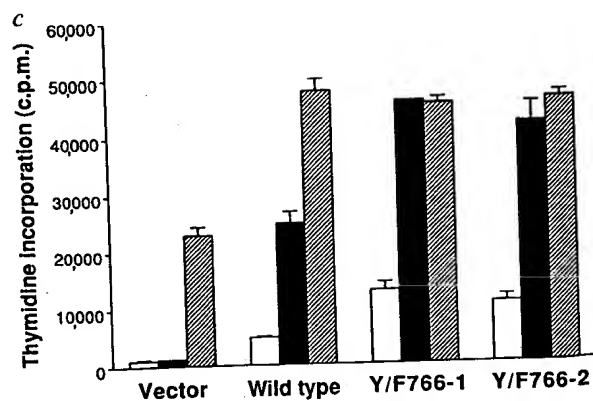
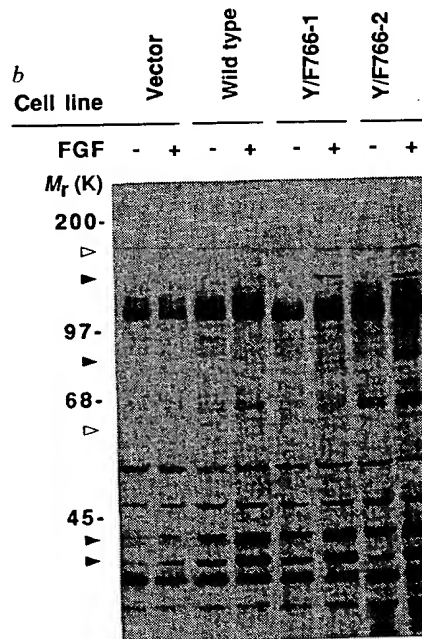


FIG. 4 The Y/F766 FGF receptor mutant is an active kinase that mediates several functions of the wild-type receptor *in vivo*. a, The Y/F766 FGF receptor expressed in L6 myoblasts is autophosphorylated after stimulation with FGF. Anti-FR immunoprecipitates from unstimulated (–) and FGF-stimulated cells (+) were probed with anti-PT. Arrowhead in the upper panel indicates autophosphorylated FGF receptor. The blot was reprobed with anti-FR; the arrowhead in the lower panel indicates immunoprecipitated FGF receptor. b, Besides autophosphorylation, the Y/F766 mutant mediates ligand-dependent tyrosine phosphorylation of other cellular proteins. Lysates from the L6 cell transfectants were assayed by immunoblot with anti-PT. Closed arrowheads indicate tyrosine phosphoproteins detected after stimulation of either the wild-type or Y/F766 FGF receptor ( $M_r$  145K, 90K, 42K and 35K). Open arrowheads indicate two additional substrates that are detected after FGF stimulation of the Y/F766 receptor ( $M_r$  160K and 55K). c, The Y/F766 mutant mediates DNA synthesis in response to FGF stimulation. [ $^3\text{H}$ ]thymidine incorporation was measured in the L6 myoblast transfectants after stimulation with FGF ( $25 \text{ ng ml}^{-1}$ ). Open bars, unstimulated; solid bars, FGF-stimulated; hatched bars, serum-stimulated (10% FCS). Error bars indicate s.e.m. of triplicate samples.

METHODS. Lysates for the receptor autophosphorylation experiments and the anti-PT immunoblots were prepared as described in the legend to Fig. 2. For thymidine incorporation assays, cells were plated at equal densities on 24-well culture dishes. On day 3 the medium was changed to Q-medium. On day 4 the cells were stimulated for 18 h with the appropriate agent in Q-medium. After stimulation, cells were incubated with [ $^3\text{H}$ ]thymidine ( $1 \mu\text{Ci ml}^{-1}$ ) for 1 h at  $37^\circ\text{C}$ . Cells were then washed three times with ice-cold 5% trichloroacetic acid and solubilized for liquid scintillation counting with 0.25 N NaOH in 1% SDS.



phorylation secondary to defective activation of an intracellular serine/threonine kinase. Phosphorylation of tyrosine kinase receptors by serine/threonine kinases is thought to have a negative regulatory effect and might also explain an increase in the kinase activity of the mutant receptor<sup>18,19</sup>.

Given the proposed importance of PtdIns turnover and Ca<sup>2+</sup> mobilization in growth factor-stimulated processes and the profound effect FGF treatment of cells has on these pathways, we were surprised to find that FGF stimulated mitogenesis in cells expressing the Y/F766 mutant as well or better than in cells expressing the wild-type receptor. DNA synthesis increased significantly in all cell lines tested (Fig. 4c) and cell numbers increased in proportion to DNA synthesis (data not shown).

PLC $\gamma$  SH2 domains can bind to Tyr 992 in the C-terminal tail of the epidermal growth factor (EGF) receptor, and a C-terminal tail of a truncated EGF receptor which is also mutated at Tyr 992 still phosphorylates PLC $\gamma$  but does not activate PtdIns turnover<sup>20,21</sup>. Also, truncated EGF receptors lacking multiple potential autophosphorylation sites, including Tyr 992, do not associate with or phosphorylate PLC $\gamma$ , are defective in EGF-mediated receptor downregulation, and can transform cells<sup>22-25</sup>. The colony-stimulating factor (CSF-1) receptor can mediate mitogenesis in fibroblasts without phosphorylating or activating PLC $\gamma$  (ref. 26). Overexpression of PLC $\gamma$  gives increased PtdIns turnover in response to FGF or PDGF but does not enhance mitogenesis in response to either growth factor, suggesting that PtdIns turnover is not limiting in the mitogenic response to FGF or PDGF<sup>27,28</sup>. Our data demonstrate that increased PtdIns turnover and intracellular calcium mobilization are not required for mitogenesis in response to FGF in L6 myoblasts. Thus other signalling pathways must be involved in mitogenesis by the FGF receptor.

If not required for mitogenesis in response to FGF, what other cellular responses might be triggered by PtdIns turnover? Recent studies suggest that the activation of PLC $\gamma$  might mediate chemotaxis or cell-shape changes<sup>29</sup>. Others indicate that PtdIns turnover in response to FGF might mediate cellular differentiation during early embryonic pattern formation<sup>30</sup>. If the Y/F766 mutant does indeed have a selective signalling defect, it should be useful in establishing the role of FGF-mediated PtdIns turnover in chemotaxis, cell differentiation and other non-mitogenic cellular responses. □

## Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis

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STIMULATION of growth factor receptors with tyrosine kinase activity is followed by rapid receptor dimerization, tyrosine autophosphorylation and phosphorylation of signalling molecules such as phospholipase C $\gamma$  (PLC $\gamma$ ) and the *ras* GTPase-activating protein<sup>1,2</sup>. PLC $\gamma$  and GTPase-activating protein bind to specific tyrosine-phosphorylated regions in growth factor receptors<sup>3-9</sup> through their *src*-homologous SH2 domains<sup>7,8,10,11</sup>. Growth factor-induced tyrosine phosphorylation of PLC $\gamma$  is essential for stimulation of phosphatidylinositol hydrolysis *in vitro*<sup>12</sup> and *in vivo*<sup>13</sup>. We have shown that a short phosphorylated peptide containing tyrosine at position 766 from a conserved region<sup>14-18</sup> of the fibroblast growth factor (FGF) receptor is a binding site for the SH2 domain of PLC $\gamma$  (ref. 8). Here we show that an FGF receptor point mutant in which Tyr 766 is replaced by a phenylalanine residue (Y766F) is unable to associate with and tyrosine-phosphorylate PLC $\gamma$  or to stimulate hydrolysis of phosphatidylinositol. Nevertheless, the Y766F FGF receptor mutant can be autophosphorylated, and can phosphorylate several cellular proteins and stimulate DNA synthesis. Our data show that phosphorylation of the conserved Tyr 766 of the FGF receptor is essential for phosphorylation of PLC $\gamma$  and for hydrolysis of phosphatidylinositol, but that elimination of this hydrolysis does not affect FGF-induced mitogenesis.

To study the role of Tyr 766 in FGF receptor signalling, we generated transfected cell lines expressing either wild-type or three different FGF receptor mutants. The mutant FGF receptors included a point mutant in which Tyr 766 was replaced by a phenylalanine residue (Y766F), a control mutant in which an adjacent non-phosphorylated Tyr 776 was replaced by a phenylalanine residue (Y776F), and a double mutant in which both Tyr 766 and Tyr 776 were substituted by phenylalanine residues (Y766/776F). Wild-type or mutant FGF receptors were expressed in transfected L6 myoblasts lacking endogenous FGF receptors. Several cell lines expressing each mutant receptor were generated and characterized. These cell lines were treated with acidic FGF, lysed, immunoprecipitated with anti-FGF receptor antibodies and, after SDS-PAGE, immunoblotted with either anti-FGF receptor or anti-phosphotyrosine antibodies (Fig. 1). This experiment shows that, in response to acidic FGF, both wild-type and mutant FGF receptors undergo tyrosine autophosphorylation. We next compared tryptic phosphopeptide maps of wild-type and mutant FGF receptors. Figure 2 shows that the tryptic digest of wild-type FGF receptor contains three phosphopeptides and that phosphopeptide P1 (Fig. 2a), which contain Tyr 766 (ref. 8), is missing from the tryptic digests of FGF receptor mutants Y766F and Y766/776F.

We next examined the capacity of mutant FGF receptors to associate with and tyrosine-phosphorylate PLC $\gamma$ . Figure 3 shows that only wild-type FGF receptor and the FGF receptor Y776F mutant could associate with (Fig. 3a, b) and mediate tyrosine phosphorylation (Fig. 3c, d) of PLC $\gamma$  in living cells. In contrast, no tyrosine phosphorylation of PLC $\gamma$  was detected in cells expressing either Y766F or Y766/776F mutants. As growth factor-induced tyrosine phosphorylation of PLC $\gamma$  is

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